



RESEARCH PAPER

Differential distribution of the cognate and heat-stress-induced isoforms of high M_r *cis-trans* prolyl peptidyl isomerase (FKBP) in the cytoplasm and nucleoplasm

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Abstract

Wheat root tips express a 73 kDa cognate isoform and a 77 kDa heat-shock-induced isoform of peptidyl prolyl *cis-trans* isomerase (FK506 binding protein; FKBP) that is part of a chaperone complex with hsp90. The 73 kDa and 77 kDa FKBP have very similar sequences, differing primarily in the N- and C-terminal 20 amino acids. In order to define the potential functional roles of these proteins, the 73 kDa and 77 kDa FKBP were localized in root tips using antigen-affinity purified antibodies as a probe. The cognate 73 kDa FKBP is localized in the cytoplasm and appears enriched around the periphery of the early vacuole and vesicles exiting the *trans*-Golgi. Parallel assays with antibodies directed against tonoplast aquaporin and pyrophosphatase confirmed the association of FKBP with an early vacuole compartment. Sucrose gradient centrifugation analysis of root tip lysates also showed that 73 kDa FKBP is co-fractionated with tonoplast aquaporin and V-ATPase in a light compartment near the top of the gradient. Heat-shock treatment of root tips induces the accumulation of 77 kDa FKBP while the abundance of 73 kDa FKBP remains constant. Quantitative EM immunogold assays of the intracellular distribution of FKBP over an 8 h heat-shock time-course showed that FKBP is initially present in the cytoplasm, but is transported into the nucleus where it accumulates in the nucleoplasm and into specific subnuclear domains. The results of this study show that the intracellular distribution of the high M_r FKBP in

wheat root tips differs at normal and elevated temperatures, indicating different functional roles for the FKBP isoforms.

Key words: *cis-trans* prolyl isomerase, FK506-binding protein (FKBP), Golgi, nucleus, prevacuole, provacuole, vacuole.

Introduction

In plants, *cis-trans* prolyl isomerases (PPIases) belonging to the cyclophilins, FKBP and parvulins have been cloned (Boston *et al.*, 1996; Landrieu *et al.*, 2000). Cyclophilins were found in the cytosol, chloroplast and mitochondria (Gasser *et al.*, 1990; Breiman *et al.*, 1992; Lippuner *et al.*, 1994; Luan *et al.*, 1994, 1996; Fulgosi *et al.*, 1998). The PPIases catalyse the isomerization of the peptide bond between a proline and the peptide residue. The *cis-trans* inter-conversion accelerated by PPIases is significant for the final protein structure because *cis* proline introduces bends within the protein. FK506 binding proteins (FKBP) are found in all classes of organisms, having multiple roles in the cells, the best known being receptors for medically important immunosuppressors. Although most cellular functions of different FKBP are still unknown, recent results obtained in plants and animals indicate that these proteins are master regulators in the control of development (for reviews see (Harar and Faure, 2001; Breiman and Camus, 2002).

Plant FK506 binding proteins (FKBP) have been isolated from several species such as wheat FKBP73 (Blecher *et al.*, 1996; Aviezer *et al.*, 1996), *Arabidopsis*

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62 kDa ROF1 (Vucich and Gasser, 1996), *Arabidopsis* and *Vicia faba* FKBP15 (Luan *et al.*, 1996), *Arabidopsis* *pas1* encoding a 70 kDa FKBP (Vittorioso *et al.*, 1998), maize FKBP66 (Hueros *et al.*, 1998), and the wheat heat-stress-induced FKBP77 (Kurek *et al.*, 1999). As in animals, the plant FKBP s are directed to different compartments in the cell.

The plant high molecular weight FKBP s differ from the small FKBP12 in that they contain one or more FKBP12-like domains, a tetratricopeptide repeat (TPR) domain and a C-terminal domain that, in most cases, binds calmodulin. They are cytosolic, showing high sequence and structural similarity among themselves and with the mammalian FKBP52 (Blecher *et al.*, 1996; (Harar and Faure, 2001). The mammalian FKBP52, together with the hsp90, are part of the mammalian steroid receptor complex. Upon hormone binding the hsp90 soluble receptor complex dissociates and the receptors reaching the nucleus dimerize allowing them to act as transcription factors.

Most of FKBP52 localizes by indirect immunofluorescence to the nucleus with the rest being localized to microtubules in the cytoplasm (Czar *et al.*, 1994). FKBP52 has been implicated in targeting the nuclear localization signal (NLS)-directed movement of the glucocorticoid receptor along microtubular pathways to the nucleus (Pratt, 1998). The nuclear localization signal was suggested as a potential site of immunophilin interaction with the receptor when it was noted that FKBP52 contains a conserved sequence of negatively charged amino acids complementary to the receptor nuclear localization signals (Picard and Yamamoto, 1987; Czar *et al.*, 1995). Recently, it was found that FKBP52 binds to dynein suggesting that FKBP52 targets receptor movement toward the nucleus by determining its attachments to retrograde dynein motor protein (Silverstein *et al.*, 1999; (Galigniana *et al.*, 2001). Taken together these observations lead to the function of FKBP52 in targeting receptor movement (Pratt *et al.*, 1999).

The wheat FKBP73 and wheat FKBP77 possess, close to their N-terminus, one PPLase domain with high similarity to the FKBP12, two additional FKBP12-like domains showing less sequence conservation, three TPR repeats forming the TPR domain, and a C-terminal calmodulin-binding domain. The wheat FKBP s (wFKBP) were shown to interact with HSP90 and to bind calmodulin, being able to exchange the mammalian FKBP52 in the steroid receptor complex (Reddy *et al.*, 1998; Kurek *et al.*, 2002).

FKBP73 and FKBP77 are highly similar proteins differing in primary sequence at the amino and carboxyl ends. The FKBP73 is expressed in immature tissues and reproductive organs, while FKBP77 is induced by heat shock in the same tissues. The close similarity of these two proteins and their expression occurring in similar tissues and developmental stages presents an opportunity to

compare two closely related proteins that are differentially regulated. The presence of FKBP-binding proteins in multiple cellular compartments supports diverse roles for these proteins in both signalling and protein processing.

Materials and methods

Plant material

Wheat (*Triticum aestivum* L.) seeds cv. Jackson were surface-sterilized and imbibed overnight. The imbibed seeds were further incubated on wet filter paper for 1 d to obtain the root tips from 2-d old seedlings.

Anti-FKBP73 and 77 peptide antibodies

Isoform-specific antibodies for both the FKBP73 and FKBP77 were elicited by immunization with the unique carboxyterminal sequences (-CSRMTKPSAEESKA for FKBP73 and -CAKWRKTENAAGK-QEAPMASDSTA for FKBP77) peptide, produced in-house with an Applied Biosystems peptide synthesizer using standard supplier's procedure and coupled to malamide activated haemacyanin by the added amino-terminal cysteine. The immunogen was used to inject rabbits and sera obtained on the vendor's schedule (Spring Valley, Sykesville, MD, USA). Affinity purified anti-FKBP73 and 77 IgGs were obtained by immobilizing the peptides on an activated sepharose support (Sulfolink, Pierce Biotechnology, Rockford, IL) and passing the sera over the column. The immobilized antibodies were displaced by low pH, dialysed against TBS and quantified for concentration. The monospecific anti-FKBP73 and -FKBP77 peptide antibodies yielded only one band on SDS/PAGE immunoblots and did not exhibit any cross-reactivity with the other FKBP isoform.

One- and two-dimensional SDS/PAGE immunoblot assays and sucrose gradients

To examine the polypeptide distribution of root tip proteins after a time-course of heat stress (37 °C) treatment, 2 d whole seeds were stressed in an incubator and, at different time points, root tips were excised and ground in a mortar and pestle in 2 ml g⁻¹ of tissue using the SDS sample buffer (Laemmli, 1970) containing 6 M urea substituting for glycerol. The samples were heated to 70 °C for 10 min and clarified by 5 min centrifugation in a microfuge. The samples were fractionated on 10% SDS/PAGE gels and blotted onto nitrocellulose membranes for antibody binding analysis as described for the sucrose gradient analysis.

For sucrose gradient analysis root tips from 2 d seedlings were extracted by gentle maceration with a mortar and pestle in 50 mM HEPES, 250 mM sorbitol, 6 mM EDTA, 1 mM DTT, and 0.1 mM PMSF pH 7.4. The cell wall fragments and other large materials were removed by centrifugation at 1000×g for 5 min at 4 °C. The resulting supernatant was loaded on a continuous 10–40% (w/v) sucrose gradient in the extraction buffer. The gradients were centrifuged overnight (14 h) in a Beckman SW-41 rotor at 39 000 rpm at 4 °C. The resulting gradient was fractionated in 0.7 ml fractions, divided in half, with one set precipitated with TCA and the other half retained frozen as replicates. The TCA pellet was washed with acetone and the pellet was solubilized in 100 µl of SDS sample buffer. SDS/PAGE fractionation of the protein pellets and electrotransfer of proteins to nitrocellulose blots were accomplished by standard procedures (Laemmli, 1970; Towbin *et al.*, 1979). Immunological labelling of the blots was accomplished by blocking the blots in 3% gelatin in TBST and then incubating the blots overnight in a dilution of the primary antibody in 1% gelatin in TBST. The anti-FKBP serum was diluted 1:5000, the anti- α -TIP was diluted 1:10 000, the culture supernatant of V-ATPase monoclonal

antibody 7A5 (gift of Dr H Sze, University of Maryland, College Park) was diluted 1:300, the anti-BiP was diluted 1:2500 (gift of Dr R Boston, North Carolina State University), and anti-yeast-hsp90 (Sigma) was diluted 1:1000. The detection of the bound antibodies was accomplished by indirect labelling with anti-IgG-peroxidase and detection by chemiluminescence. Visualization and recording of labelled bands was accomplished by exposure to Kodak OMAT X-ray film.

For floatation gradient the same procedure was followed except the initial lysate sample was made up to 45% w/v sucrose and loaded in the bottom of the tube prior to overlying the sample with the continuous sucrose gradient.

Two-dimensional gel electrophoresis and immunoblot

Two-dimensional IEF/SDS PAGE analysis was accomplished using the method of Remy and Ambard-Bretteville (1987). Lysate samples were prepared by grinding the wheat root tissue with 7 vols (w/v) of a urea/Triton X100/mercaptoethanol extraction buffer followed by centrifugation in a microfuge to remove cellular debris. The samples were fractionated with 1.5×13 cm tube gel containing wide range (pH 3.5–9.0) ampholytes at 1000 V for 2 h. The second dimension was accomplished by equilibrating with tube gel the extraction buffer containing SDS and electrophoresis as in single dimension SDS gels. The fractionated proteins were transferred to a PDVF membrane and labelled with anti- α -TIP antibodies as described for single dimension SDS/PAGE.

Electron microscopy and immunocytochemical assays

The 2 d root tips were fixed and embedded by the standard protocols of this laboratory (see Herman and Melroy, 1990, for detailed protocols). Briefly, this consists of fixation in 4% formaldehyde and 2% glutaraldehyde in 0.5 M phosphate buffer pH 7.4. The samples were divided in half and some of the root tips were post-fixed with 1% aqueous OsO₄ for conventional EM observations while the portion was processed without osmium for use in immunocytochemical assays. The root tips were dehydrated in graded ethanol and embedded in LR White resin. For immunocytochemical assays, thin sections mounted on grids were labelled with immunoaffinity purified rabbit anti-FKBP73 antibodies, rabbit anti- α -TIP (a tonoplast aquaporin, provided by Dr Maarten Chrispeels, UCSD, San Diego, CA), and mouse anti-yeast-hsp90 (Sigma) diluted in TBST. Ultrathin sections of the osmicated root tips were stained with 5% aqueous uranyl acetate and 3% basic lead citrate prior to viewing.

The resulting material was visualized with a Phillips 400T electron microscope and all images were captured with an axial mounted AMT CCD camera interfaced to a Macintosh G3 computer. Images were initially captured with IP Lab spectrum software and later processed for publication with Adobe Photoshop and Illustrator.

Quantitative immunogold assays were accomplished by capturing a large number of images with the CCD camera at constant magnification. Gold particle density per unit area was determined and tested for validity by statistical analysis using analysis of variance.

Results

FKBP73 has previously been shown to be present in root tips (Aviezer *et al.*, 1996) assayed by immunoblots using holo-FKBP73 antibodies while FKBP77 was induced by heat stress. Lysates were prepared from root tips of germinated seeds (20 °C) and after 1, 2, 4, 6, and 8 h of heat stress at 37 °C. The lysates fractionated by SDS/

PAGE were transferred to PDVF membranes and parallel blots were labelled with anti-FKBP73, anti-FKBP77 and anti-hsp90 antibodies. A parallel blot was stained with amido black to confirm correct loading and concentration of protein for each time point. Using an affinity-purified FKBP73 isoform-specific antibody, a single FKBP73 band was labelled in immunoblots of SDS/PAGE fractionated root lysates, which continues to be present throughout the course of an 8 h heat stress with little apparent change in abundance (Fig. 1).

By contrast, the FKBP77 isoform is not present at 20 °C, but is induced and accumulated during the course of 8 h of heat stress at 37 °C. The FKBP77 band appears after 2 h and a maximal level is observed after 6 h of heat stress. The weak band initially observed after 1 h of heat stress is slightly higher in M_r than the primary FKBP77 band and this band continues to be present during the 8 h time-course. Whether this band represents a modified early form of FKBP77 whose sequence possesses potential modification sites or is an immunological artefact induced by heat stress and labelled with antigen affinity purified antibodies remains unresolved and the subject for further investigations.

Hsp90 is a partner protein of both FKBP73 and FKBP77 in a cytoplasmic chaperone complex (Reddy *et al.*, 1998). Hsp90 is highly conserved in eukaryotes and a commercially available monoclonal antibody directed against yeast hsp90 is cross-reactive with plant, animal as well as yeast hsp90. A parallel immunoblot labelled with anti-yeast-hsp90 monoclonal antibody showed a single band at 20 °C that appears to increase in concentration slightly during the heat stress. A weakly labelled band of slightly smaller M_r than hsp90 appears after 2 h of heat stress and continues to accumulate through 8 h reaching maximal apparent level after 6 h. Like the weak band observed with FKBP77 antibody labelling, it is unresolved whether this band represents a modified form of hsp90, another isoform, or an assay artefact.

At 20 °C FKBP is localized with Golgi-secretion vesicles and the prevacuole

In order to determine the intracellular distribution of FKBP73 in the root tip cells, plastic sections were labelled with recombinant FKBP73 antigen-affinity purified IgGs as the primary probe. The holo-FKBP antibody was used for all EM assays as the two antipeptide antibodies that are isoform-specific did not prove to be good probes for root tip tissue fixed and embedded in plastic. The initial examination of the intracellular labelled sites showed a punctate pattern of locules in the cytoplasm. High magnification examination of the labelled sites indicated that the gold particles were associated with small membrane vesicles associated with the enlarging immature vacuole. Figure 2 shows an example of the labelling pattern where the gold particles are associated

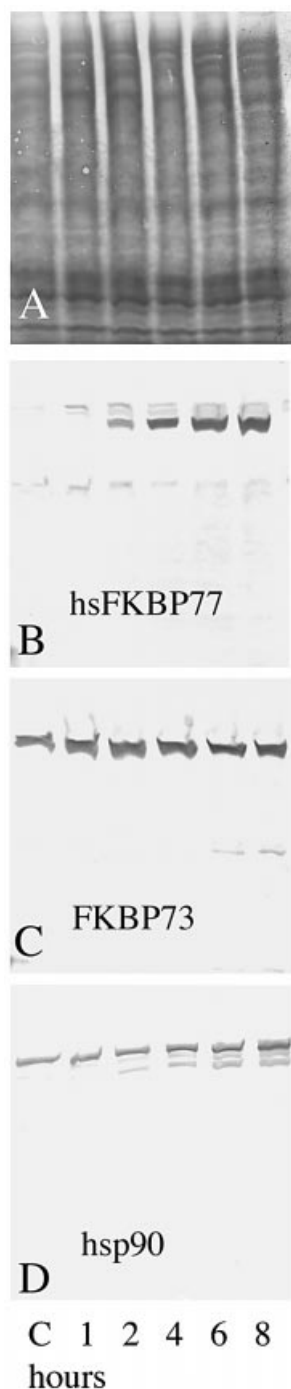


Fig. 1. SDS/PAGE immunoblot analysis of root tip lysates from samples taken during a heat shock time-course shows the induction of FKBP77 accumulation. (A) Total protein visualized by amido black stain. (B, C) The abundance of FKBP77 and FKBP73, respectively, assayed with isoform-specific antibodies. (D) hsp90 abundance assayed with a monoclonal antibody elicited against yeast hsp90 that is cross-reactive with wheat hsp90. The immunoblot assay shows that FKBP73 abundance is very similar during the 8 h time-course of the heat shock, while FKBP77 is induced, beginning to accumulate after 1 h and continues to accumulate.

with vesicles aggregating to form the vacuole (Fig. 2, PV). The gold particle labelling was restricted to immature vacuoles in the initial stages of assembly by vesicular aggregation and was not associated with larger, but still immature vacuoles abundant in the root tips (Fig. 2A, V). The anti-FKBP73 antibody labelled the vesicles apparently secreted from the *trans*-Golgi (Fig. 2A, B; G, arrows) but did not label the cisternal stack of any of the Golgi.

The pre/early vacuole is labelled with antibodies against α -TIP

The organelles labelled by the anti-holo-FKBP73 antibody appear to be the immature vacuoles. In order to characterize and identify the vesicles that are labelled with the anti-FKBP73 antibody, antibodies elicited against the tonoplast protein α -TIP was used to label 2-d-old root tip sections. α -TIP antibody was originally elicited against the major aquaporin of maturing legume seeds (Johnson *et al.*, 1990), but subsequent experiments have shown that there are TIPs in the meristem that share an epitope(s) with the seed-specific α -TIP and this antibody has proved to be a useful probe to identify tonoplast in forming vacuoles (Paris *et al.*, 1996). The α -TIP antisera yielded highly specific labelling of the membranes of the immature vacuole (Fig. 3, PV, arrows) while the vacuoles (V) forming the prevacuole were labelled less intensely.

Sucrose gradient analysis of root tip lysate

In order to obtain correlative observations on the distribution of FKBP and with other marker proteins, wheat root tip lysates derived from 2 d germinated seeds were fractionated on continuous sucrose gradients. The resulting fractions were concentrated and analysed by SDS/PAGE-immunoblot assays with affinity-purified anti-holo-FKBP, anti- α -TIP, anti-V-ATPase, and anti-BiP as probes (Fig. 4A). The affinity purified anti-FKBP antibody labelled a 73 kDa band consisting of four fractions of low density near the top of the gradient. The additional markers for the tonoplast consisted of a polyclonal antibody directed at α -TIP and a monoclonal antibody directed at the V1 sector of the V-ATPase. The α -TIP antibody labelled the same low-density fraction as the FKBP. The two distinct bands observed in each fraction may be a consequence of the protein's tendency to dimerize as a result of sucrose-induced dehydration of the membrane fractions. By contrast, the V-ATPase distribution is different from the pattern of labelling of both the anti- α -TIP and anti-FKBP patterns. V-ATPase is present in the low-density fractions that contain the α -TIP and FKBP proteins, but the majority of V-ATPase is present in higher-density fractions as previously observed in similar gradient of oat root tips (Herman *et al.*, 1994). An additional band was labelled with the affinity-purified anti-FKBP in the middle of the gradient. The size of this

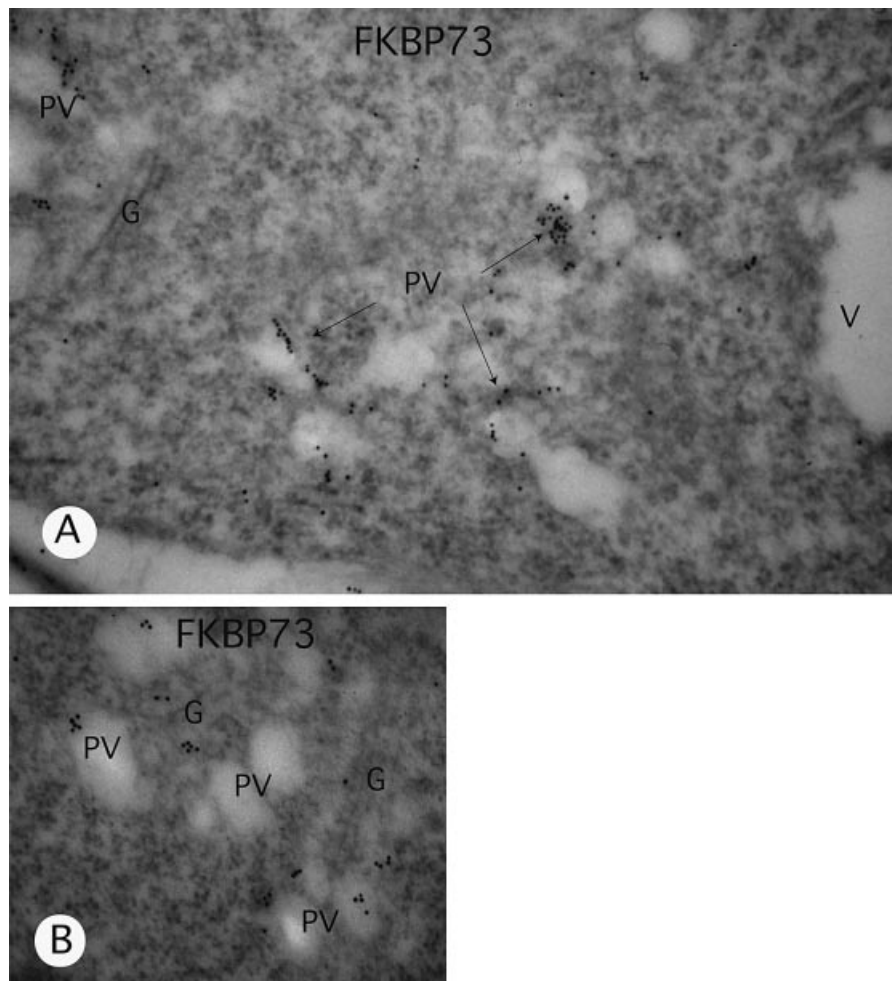


Fig. 2. Immunocytochemical localization of FKBP73 in a root tip cell. (A) The anti-FKBP73 labels the vesicles constituting the prevacuole (PV) while not labelling adjacent vacuoles (V). Prevacuole vesicles associated with the *trans*-Golgi (G) are also labelled while the cisternae of the Golgi is not labelled. (B) Immunogold assay showing the association of FKBP73 with the prevacuole vesicles (PV) apparently originating from two Golgi (G). Note the immunogold labelling appears to be associated only with the prevacuole vesicles and not with the cisternae of the Golgi.

polypeptide is consistent with the expected size of the 12 kDa ER-resident *cis-trans* prolyl isomerase. All *cis-trans* prolyl isomerases share conserved sequences consisting of modules that possess the FK506 binding site and the rotamase (prolyl isomerase) active site. Probing a replicate blot with the ER resident protein BiP as a marker indicates that the weakly labelled 12 kDa FKBP-cross-reactive band is likely to be an ER protein. ER labelling was not observed in the EM-immunogold assays and this may be due to a low level of apparent labelling evident on the blots that have a higher signal amplification due to the enzyme-linked assay method.

2D gel analysis supports the presumed dimerization of TIP

The double band labelled by the α -TIP antibody could indicate either a dimerization of the protein or two

distinct gene products. The dimerization of aquaporins such as TIP has been previously observed and may be induced by the dehydration of the membranes by sucrose. In order to provide an alternate form of analysis, 2 d root tips were analysed by two-dimensional gel electrophoresis using a primary extraction in the presence of 8 M urea that should impede protein aggregation artefacts. Figure 4b shows the resulting blots with parallel blots stained for total protein and a second blot labelled with the α -TIP antibody. The total protein blot shows a multitude of protein spots indicating the separation technique was effective at separating the root tip polypeptides. The α -TIP antibody labelled a single elongated protein band (26 kDa) that is spread by isoelectric point, but not by M_r , supporting the suggestion that the two bands, cross-reactive with anti- α -TIP in the sucrose gradient fractions, are a biochemical artefact.

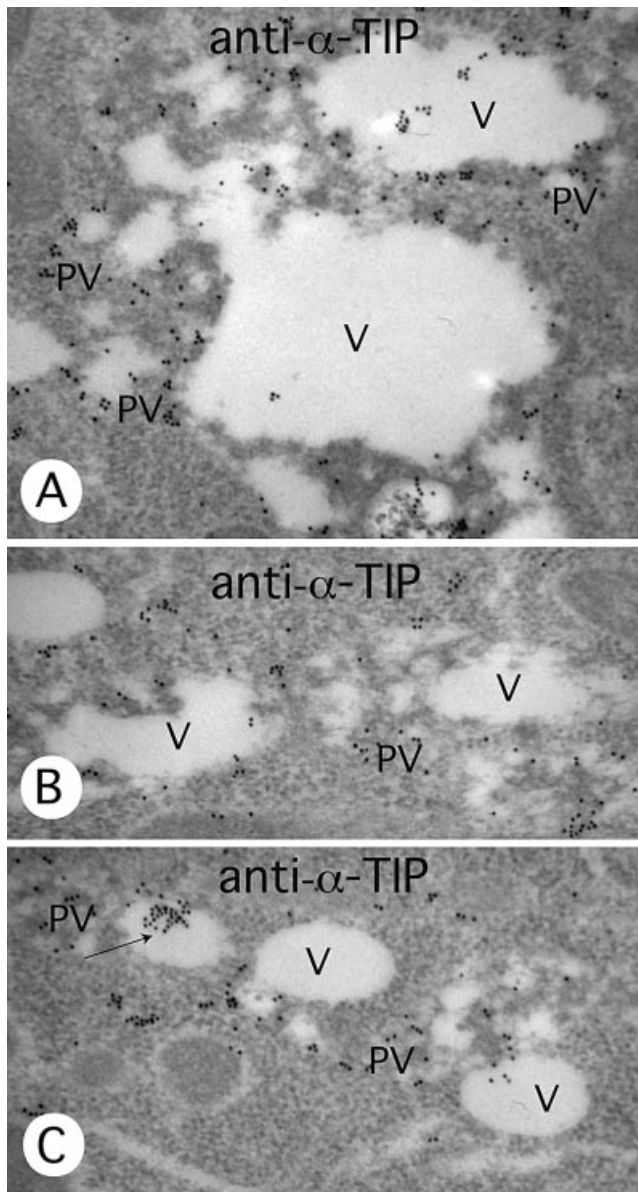


Fig. 3. Immunogold localization of the aquaporin cross-reactive with the α -TIP antibody. Note that the prevacuole vesicles (PV) are enriched in α -TIP while the vacuoles (V) that result from the aggregation of the prevacuoles are labelled with few gold particles. This indicates that α -TIP association with the forming vacuole is transient with the aquaporin removed once the maturation of the vacuole is initiated.

Floatation gradient indicates FKBP73 is not tightly bound to provacuole membranes

FKBP73 and the associated cytoplasmic chaperone complex are presumed to be cytosolic proteins, yet both EM immunocytochemistry and isopycnic sucrose gradient assays indicate that FKBP73 is associated with the tonoplast membranes of the earliest stages of vacuole assembly. In order to test whether the FKBP73 is tightly associated with a membrane compartment, cellular lysates

were assayed by sucrose floatation gradient. Isopycnic fractionation after loading the cellular lysate in the bottom of the centrifuge tube with 40% (w/v) resulted in all of the anti-FKBP73 antibody cross-reactivity remaining in the soluble phase and not fractionating with any membranes that would rise in the gradient (Fig. 4c).

FKBP77 accumulates in the nucleus as the consequence of heat-stress

Labelling thin sections of 37 °C heat-stressed root tips with affinity-purified anti-holo-FKBP antibody indicated that heat stress induced a different distribution of FKBP. Unlike cells grown at 20 °C where the gold label was exclusively cytoplasmic and associated with the provacuole vesicles, the nuclei of the heat-stressed root tips consistently exhibited a much higher level of gold particle label compared with the control. Figure 5A and B show an example that illustrates the intranuclear labelling of root tip nuclei of 20 °C at zero time and after 8 h at 37 °C.

In order to characterize the accumulation of FKBP in the nucleus, quantitative immunogold analysis was conducted on a set of samples of a heat-stress time-course. Thin sections prepared from 0, 1, 2, 4, 6, and 8 h of 37 °C heat-stressed root tips were simultaneously labelled with affinity purified anti-FKBP IgGs using the same dilution, solutions and incubation time to standardize the assay. Large areas of cytoplasm and numbers of nuclei were imaged for each time point using the EM with its CCD imaging system. The gold particle density was counted per unit area and statistically valid data assembled. The resulting data are shown in Fig. 6. Comparison of the cytoplasmic and intranuclear concentration of gold particles indicate that FKBP levels rise rapidly after 37 °C heat-stress induction with the protein initially accumulating in the cytoplasm (1–4 h of heat-stress). FKBP is then gradually accumulated in the nucleus with its density exceeding that in the cytoplasm after 4 h of heat-stress.

Double labelling indicates FKBP colocalizes with a subset of hsp90 distribution

Both FKBP73 and FKBP77 are partner proteins in the cytoplasmic chaperone complex with hsp90 (Reddy *et al.*, 1998). Hsp90 should have a cellular distribution that overlaps that of FKBP. The nuclei from 20 °C and 37 °C heat-stressed root tip cells were examined using an anti-hsp90 monoclonal antibody and anti-FKBP IgG with double labelling assays using 5 and 10 nm gold particle sizes. Little hsp90 or FKBP was labelled in the nuclei of 20 °C cells (Fig. 7A). By contrast, in 37 °C heat-stressed cells, the nuclei contains abundant label representing sites of intranuclear hsp90 and FKBP (Fig. 7B, C).

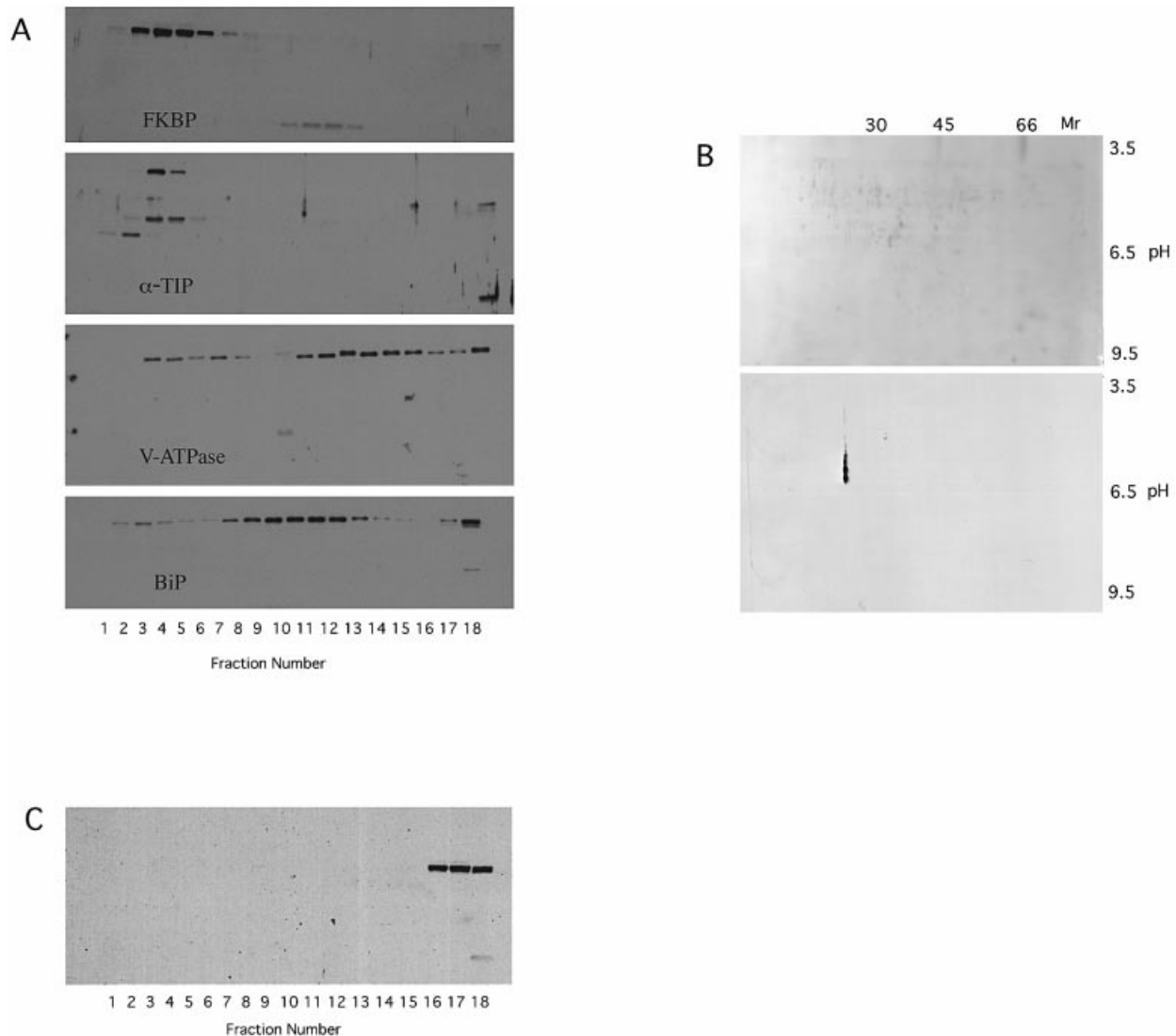


Fig. 4. (A) The fractionation of a root tip lysate on a continuous 10–40% (w/v) sucrose gradient results in the separation of the prevacuolar compartment from the tonoplast. The FKBP73 and aquaporin cross-reactive with α -TIP antibody are present in the same low-density fraction indicating that these two proteins are sequestered together consistent with the results of the immunogold assays shown in Figs 2 and 3. The prevacuole compartment also contains a fraction of the V-ATPase although a majority of the proton transporter is present in the higher density tonoplast fraction. Also shown is the distribution of the ER indicated by the distribution of the ER chaperone BiP. (B) A two-dimensional IEF/SDS-PAGE analysis of a wheat root tip lysate and the immunoblot assay with the α -TIP antibody. The upper panel shows the α -TIP immunoreactive protein is a dimer; however, using a denaturing (8 M urea) lysis to prepare material for the two-dimensional analysis in the lower panel results in a single spot suggesting that the apparent dimerization of the α -TIP cross-reactive protein is an artefact of preparing lysates for isopycnic gradient analysis. (C) The results of a floatation gradient on the isopycnic distribution of FKBP. Loading the lysate on the bottom on the gradient results in a retention of the immunoreactive FKBP in the high density fraction indicating that FKBP does not remain associated with a membrane that can be induced to float through the gradient.

Discussion

FKBP73 and FKBP77 are highly similar proteins differing primarily in sequence differences in the amino- and carboxy-terminal domains. The results presented here indicate that there are two distinct localization sites for the cognate FKBP73 and the heat-stress-induced FKBP77, the provacuoles and nucleus, respectively. The difference in localization indicates that FKBP73 and FKBP77 have quite distinct functions involved in vacuole ontogeny at

room temperature and some other intranuclear function as the consequence of heat-stress. FKBP73 and FKBP77 are constituents of the cytoplasmic chaperone complex with hsp97, hsp70 and p23 and it is likely that it is the function of this complex that is significant in these roles. The cytoplasmic chaperone complex (CCH) is a conserved feature of eukaryotic cells being found in animal and yeast cells (Pratt and Toft, 1997) as well as in plants (Pratt *et al.*, 2001). This complex has many described and presumed

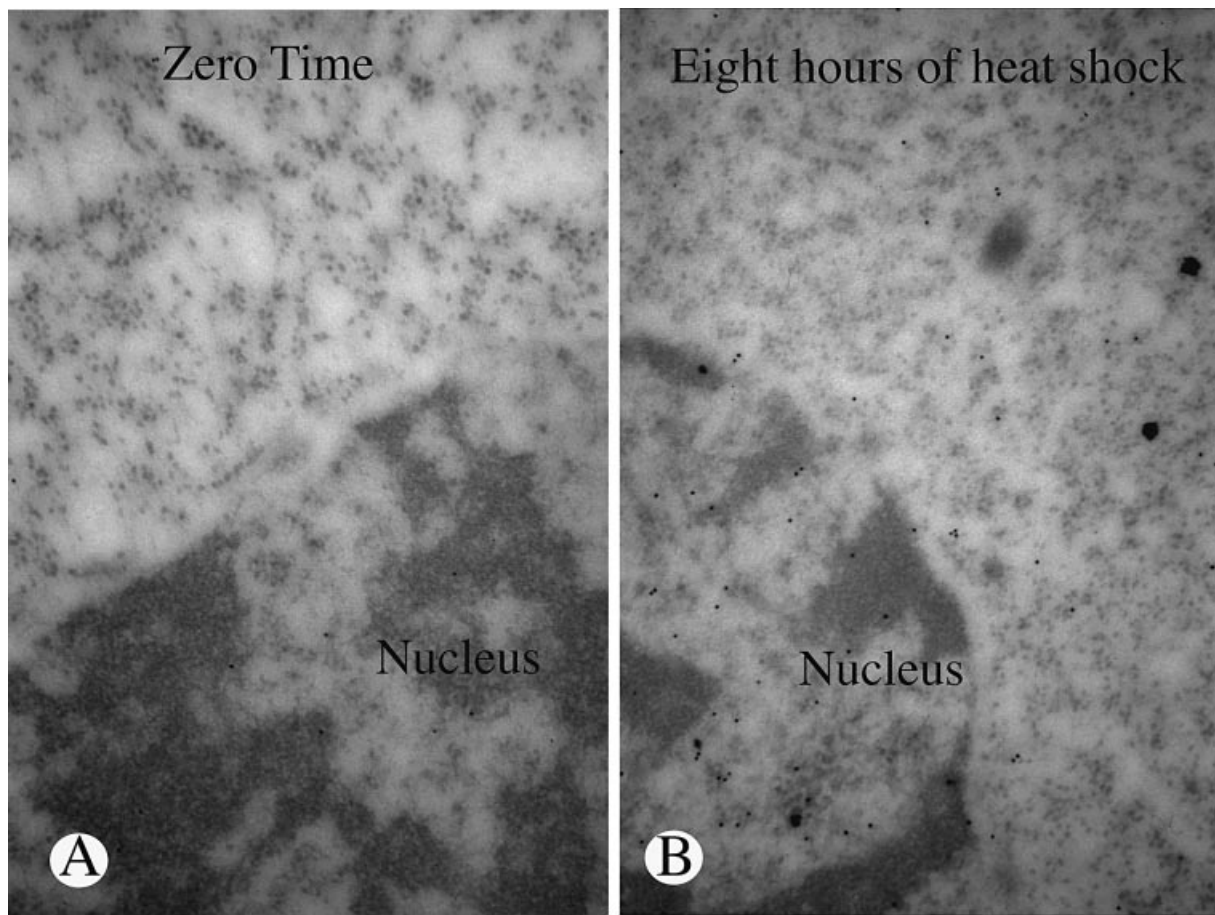


Fig. 5. Immunogold localization of FKBP in the nucleus of root tips grown at 20 °C and compared with root tips heat shocked at 37 °C for 8 h. At 20 °C there is very little labelling of the nucleoplasm (A) while after an 8 h incubation at 37 °C (B) there is abundant intranuclear labelling with anti-FKBP antibodies.

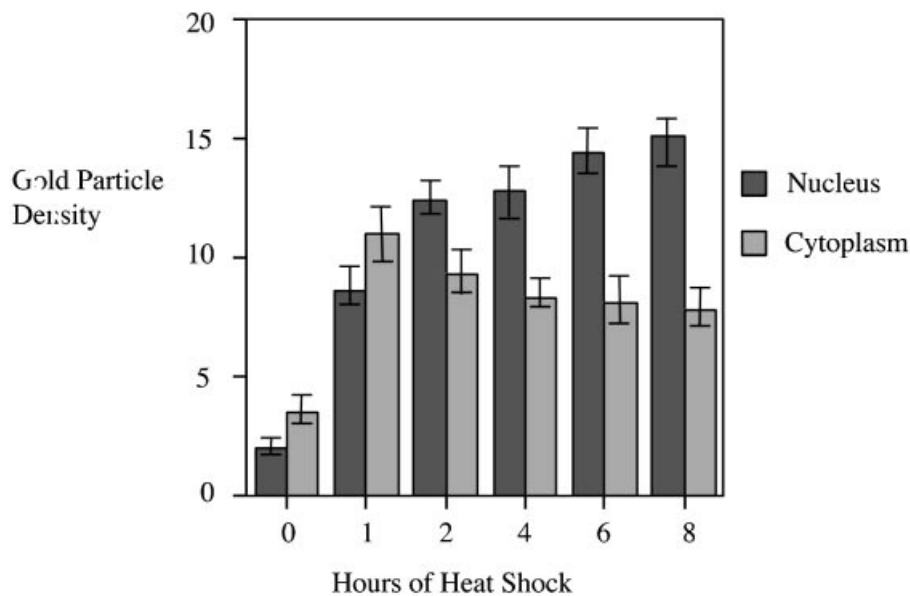


Fig. 6. Quantitative immunogold assay of the accumulation of FKBP in the nucleus of heat-stressed root tip cells. The cytoplasmic and nuclear concentration of gold particles was counted in a large sample of root tip sections during the time-course of heat stress and accumulation of FKBP77. The gold particle shows that the concentration of FKBP initially rises in the cytoplasm and then gradually declines as the protein is accumulated into the nucleus.

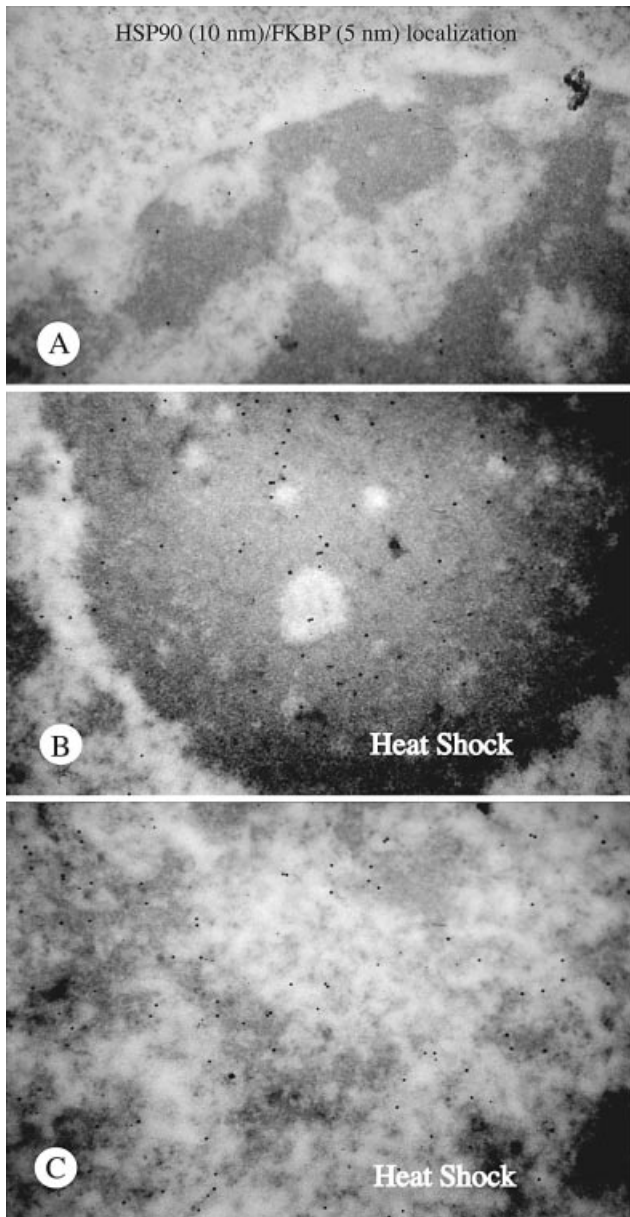


Fig. 7. A double-labelling experiment is shown that was performed with antibodies specific for FKBP and hsp90 to assay the comparative distribution of these two proteins. FKBP is a member of a cytoplasmic chaperone complex with hsp90 and therefore it would be expected that at least a portion of both of these proteins should be co-localized. Double labelling with affinity purified antibodies against holo-FKBP (5 nm gold) and anti-yeast hsp90 (10 nm gold) shows scant intranuclear labelling with both antibodies in samples from root tips grown at 20 °C (A). However, after 8 h at 37 °C the concentration of both hsp90 and FKBP rises in the root tip cell nucleus. Hsp90 is abundant in the nucleolus (B), while both FKBP and hsp90 are localized, often together, in the nucleoplasm (C).

roles (Pratt, 1998; Buchner, 1996) in assisting in the folding/processing of proteins and in signal transduction processes (Pratt and Toft, 1997; Fruman *et al.*, 1994).

Most plant cells contain prominent vacuoles that occupy much of the intracellular space. The vacuole is a post-

Golgi product of the secretory system that also sequesters inclusions derived from the cytoplasm and extracellular space. Vacuoles are highly dynamic organelles that are synthesized *de novo* in meristemic cells and are extensively modified in response to developmental regulation and environmental changes. The primary mechanism of vacuole ontogeny appears to be assembly from Golgi-derived provacuoles that fuse together producing a prevacuole that is gradually enlarged with a further hierarchy of vesicle fusions and aggregations to form the mature vacuole. The EM-immunogold results presented in this paper show that FKBP73 is associated with the provacuole vesicles and aggregates that constitute the pre/early vacuole. FKBP73 is associated only with a transient and very immature stage of vacuole ontogeny. Fractionation of root tip lysates on continuous sucrose gradients further showed that FKBP73 is associated with a low-density membrane fraction distinct from the tonoplast fraction. This low-density fraction contains an aquaporin that is cross-reactive with α -TIP antibodies and the tonoplast proton pump V-ATPase. The presence of the V-ATPase in this low-density fraction from wheat root tips confirms a prior similar observation in fractions derived from oat root tips (Herman *et al.*, 1994). However, the FKBP73 cross-reactive proteins remain in the soluble phase in a floatation gradient, indicating that FKBP73's association with the low-density membrane is labile or alternately destroyed by high concentrations of sucrose. The presence of α -TIP and V-ATPase substantiates that FKBP73 is associated with a low-density tonoplast subfraction. Such subfractions could either represent membranes derived from a developmentally distinct stage of vacuole ontogeny or a subdomain of the tonoplast derived from fragmentation of vacuole. By correlating the sucrose gradients with the EM immunogold results it has been shown that this low-density fraction consists of pro/prevacuole membranes. The EM immunogold observations presented here show that the anti- α -TIP antibody densely labels the small provacuoles that constitute the prevacuole. This indicates that an aquaporin isoform that is immunologically cross-reactive with the α -TIP group is primarily associated with the most immature stages of the vacuole formation. Identification of this aquaporin would prove a useful stage-specific probe for the formation and assembly of the vacuole in root tip cells.

The pro/prevacuole appears to be a distinct compartment that can be defined in compositional terms. This is particularly important because the morphology of prevacuole as a electron transparent vesicle bounded by a single membrane differs little from the morphology of the mature vacuole. By analogy to yeast cell vacuoles and animal cell lysosome formation the pre/prevacuole should contain proteins involved in targeting and packaging secretory proteins. In plant cells the vacuolar targeting receptor BP80 (Paris *et al.*, 1997; Ahmed *et al.*, 1997,

2000) and cargo packaging protein AtELP are localized with the provacuoles exiting the Golgi and in the prevacuole. The presence the t-snare (AtPEP12p) involved in mediating pro/prevacuole fusion events shows that there is distinctive functional composition of the prevacuole (Sanderfoot *et al.*, 1998, 1999). To this pro/prevacuole-specific set of proteins the high M_r *cis-trans* prolyl isomerase (FKBP73) and the multiprotein molecular chaperone complex can be added.

Organelle assembly is a complex process that involves regulation at many different levels including gene expression, protein synthesis and co-ordination of protein deposition, processing, and assembly. There are many stages in this process where a model would predict the involvement of extrinsic proteins and enzymes to assist in the organelle maturation. A review by Herrmann *et al.* (1999) outlined the concept of helper proteins as 'outfitters, escorts and guides' in ER lumen with a role in mediating protein trafficking to the Golgi. Each outfitter, escort and guide protein plays a specific role in assembling organelles without being part of the final structure. This concept of outfitters, escorts and guides can easily be extended to include analogous and similar proteins present in the cytoplasm as well as the mitochondria and chloroplast. Among potential partner proteins in organelle assembly are members of the various protein chaperone families. Chaperones have many established roles including folding newly synthesized proteins as well as in the repair of stress-damaged proteins. Chaperones are closely associated with several signalling pathways where their interactions could be involved in co-ordinating and regulating the process of organelle assembly. The interactions of FKBP73 with other hsp and chaperones as well as its possible function in signal transduction suggests a possible role of FKBP73 and other proteins in CCH as one potential mediator in the formation of the vacuole. The presence of 73 kDa FKBP with the initial stages of vacuole formation suggests the possibility that this complex and protein may be involved in signal and regulation processes that control vacuolation. The association of FKBP73 with the prevacuole opens new lines of inquiry on how vacuole formation and vacuolation may be controlled.

The association of FKBP77 with the nucleus provides a different perspective on the function of FKBP and the associated CCH proteins. The possible function of FKBP as molecular chaperones and as components of a signalling pathway could result in essentially identical activities of FKBP73 and FKBP77 being targeted at different protein partners and substrates. The function of FKBP77 is likely to be involved in some part of the damage control mechanisms that function following heat stress, with its role likely to be within the nucleus. Although FKBP77 is apparently synthesized in the cytoplasm where it transiently accumulates, it is probable that this is the synthesis and transport stages of FKBP accumulation with

its stress-related function occurring after the protein enters the nucleus. The intranuclear distribution of FKBP and other stress-related proteins is likely to prove significant in regulating and preserving transcriptional capability in response to abiotic stress. The partner proteins and substrates for both FKBP73 and FKBP77 may prove to be significant in vacuole ontogeny and in the heat-stress response.

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